

SNPs and Interaction Analyses of Noelin 2, Myocilin, and Optineurin Genes in Japanese Patients with Open-Angle Glaucoma

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PURPOSE. To evaluate the noelin 2 gene as a disease-causing factor for open-angle glaucoma (OAG) and the interactions between the noelin 2 (*OLF2*), optineurin (*OPTN*), and myocilin (*MYOC*) genes.

METHODS. *OLF2* was analyzed in 770 Japanese subjects including 215 patients with elevated intraocular pressure (IOP), 277 with normal IOP, 38 with juvenile open-angle glaucoma, and 240 control subjects. Two single-nucleotide polymorphisms (SNPs) in *OPTN* (c.412G→A and c.603T→A) and one SNP in *MYOC* (c.227G→A) were examined. Single genes were investigated by univariate analysis and the gene-gene interactions by logistic regression analysis. Associations between genotypes and clinical characteristics at the time of diagnosis were examined.

RESULTS. In *OLF2*, 12 sequence variants were identified in 770 Japanese subjects. Arg144Gln (exon 4) was identified in two (0.3%) of the patients and in none of the control subjects. Combinations of *OLF2*/517A and *OPTN*/412A or *OLF2*/1281T and *OPTN*/412A were associated with patients with elevated IOP ($P = 0.018$ or $P = 0.012$, respectively). The combination of *OLF2*/317G and *OPTN*/603A was significantly associated with elevated IOP ($P = 0.018$). No significant association was detected between SNPs in *OLF2* and in *MYOC*. Patients with normal IOP and with *OLF2*/678A+*OPTN*/412G or *OLF2*/1281C+*OPTN*/412G had significantly worse visual field scores ($P = 0.022$ or 0.030 , respectively).

CONCLUSIONS. The Arg144Gln mutation in *OLF2* is a possible disease-causing mutation in Japanese patients with OAG. Common polymorphisms in *OLF2* and *OPTN* may interactively contribute to the development of OAG, indicating a polygenic etiology. (*Invest Ophthalmol Vis Sci.* 2006;47:5368–5375) DOI:10.1167/iovs.06.0196

Glaucoma is the second leading cause of blindness worldwide, and is estimated to affect more than 60 million people. Open-angle glaucoma (OAG), the most common form, is present in almost 2% of the world's population older than 40 years.¹ Glaucoma includes a group of conditions that is characterized by progressive optic neuropathy with visual field changes corresponding to the damage of the retinal nerve fibers. These changes are usually associated with an elevation of intraocular pressure (IOP). Elevated IOP is generally accepted to be a major risk factor for glaucomatous changes.²

Genetic factors also play a major role in the etiology of OAG.³ The first gene to be characterized was the trabecular meshwork-inducible glucocorticoid response (*TIGR*) gene on 1q.⁴ The *TIGR* gene was mapped to the glaucoma locus GLC1A and is now known as myocilin⁵ (*MYOC*; OMIM 601652; Online Mendelian Inheritance in Man; <http://www.ncbi.nlm.nih.gov/Omim/> provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). In the eye, myocilin is expressed in high amounts in the trabecular meshwork, ciliary body, and iris and in considerably lower amounts in the retina and optic nerve head.^{6,7} Over 50 different mutations associated with the development of glaucoma have been identified in the myocilin gene in different ethnic groups worldwide.^{8–19} Mutations in the myocilin gene have been identified in 3% to 4% of all OAG cases and in 36% of cases of juvenile-onset open-angle glaucoma (JOAG) in different populations.¹⁶ Mutations in *MYOC* lead to high IOP. It has been observed that among the three exons of *MYOC*, the majority (90%) of mutations are clustered in exon 3, an olfactomedin-like domain, whereas a few (10%) are located in exon 1, a myosin-like domain, and none are in exon 2.¹⁴ Most of the mutations in *MYOC* are located in the olfactomedin domain. Olfactomedin is a secreted polymeric glycoprotein of unknown function with an evolutionarily conserved C-terminal motif.⁵

Mukhopadhyay et al.¹⁹ identified myocilin-related human proteins having a conserved olfactomedin domain using bioinformatics approaches and examined the expression patterns in the eye. Myocilin-related proteins with homology to human myocilin were selected by BLAST.²⁰ On the basis of homology to the N- and C termini of myocilin, three groups of proteins were selected: Olfactomedin 1 (noelin 1) and Pancortin isoforms, *OLF2*, and noelin 3 isoforms. The gene structures of noelin-1, -2, and -3 were determined by pair-wise BLAST²¹ analysis of the cDNA available for each gene with the retrieved genomic contig and located at 9q34.3, 19p13.2, and 1p22, respectively. Noelin 1 and 2 are expressed in the human

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retina,²² and *noelin 3* is expressed in the human retina and in trabecular meshwork cells.²³

Myocilin was found to have similar levels (60%–61%) of homology with gene products of the three *noelin* genes in the conserved ofactomedin domains. The result of phylogenetic analysis indicated that myocilin may have evolved from *noelin 2* by gene duplication followed by exon fusion.^{24,25} The coding sequences of the *noelin 2* gene (*OLFM2*) were divided into six exons spanning 82 kb of the genomic sequence. The gene is composed of a myosin-like domain (exons 1, 2, 3, and 4) and an ofactomedin-like domain (exons 5 and 6). Therefore, it is reasonable to investigate the ofactomedin domain containing proteins as potential candidates for glaucoma.

In this study, we first screened the *noelin 2* gene in Japanese patients with OAG for disease-causing mutations. OAG is a complex condition caused by multiple genes along with environmental factors that contribute to the phenotype.^{26–30} In view of the polygenic nature of OAG, it is important to explore gene-gene interactions between known candidate genes. Therefore, we also sought to determine the possible interactions between the *noelin 2*, myocilin, and optineurin (*OPTN*; OMIM 602432) genes by studying single nucleotide polymorphisms (SNPs) in a Japanese population.

METHODS

Patients and Control Subjects

A total of 770 blood samples were collected at nine institutions all over Japan. There were 215 patients with OAG who had elevated IOP, 277 with normal IOP, 38 JOAG patients, and 240 individuals with no eye disease except cataract (control subjects). No subject was related to other subjects in this study. The age at diagnosis was more than 35 years in patients with OAG and ≤ 35 years in the patients with JOAG. All patients received serial ophthalmic examinations, including IOP measurements by Goldmann applanation tonometry, Humphrey (30-2) or Goldmann perimetry, gonioscopy, and optic disc examination including fundus photographs.

In all patients, glaucoma was diagnosed according to the following criteria: the presence of typical optic disc damage with glaucomatous cupping (cup-to-disc ratio, >0.7) and loss of the neuroretinal rim; reproducible visual field defects compatible with the glaucomatous cupping; and open angles on gonioscopy. Patients with OAG with elevated IOP had an IOP >21 mm Hg at any time during the follow-up period. Patients with exfoliative glaucoma, pigmentary glaucoma, or corticosteroid-induced glaucoma were excluded. Patients with OAG who had normal IOP had glaucoma diagnosed when the untreated peak IOP was ≤ 21 mm Hg at all examinations, including the three baseline measurements and those during the diurnal test (every 3 hours from 6 AM to 12 PM); when the peak IOP, with or without medication, after diagnosis was consistently ≤ 21 mm Hg throughout the follow-up period; and when there was an absence of a secondary cause for glaucomatous optic neuropathy, such as a previously elevated IOP after trauma, a period of steroid administration, or uveitis.

Patients with more than -5.5 D of myopia were excluded. Four patients with a MYOC mutation (Ile360Asn, Ala363Thr, Thr448Pro, and Phe369Leu)³¹ and two patients with an *OPTN* mutation (His26Asp)³⁰ were also included. The procedures used in this research conformed to the tenets of the Declaration of Helsinki. Written, informed consent was obtained after the nature and possible consequences of the study were explained. When applicable, the research was approved by the appropriate institutional Human Experimentation Committee.

The mean age at the time of blood sampling was 64.7 ± 12.0 years (mean \pm SD) in the patients with OAG with elevated IOP, 55.9 ± 10.7 years in the patients with JOAG, and 60.3 ± 12.0 years in the patients with OAG with normal IOP. The control group was composed of 240 volunteers who were older than 40 years and had IOPs <20 mm Hg, normal optic discs, and no family history of glaucoma. Mean age at the

time of blood sampling was 69.7 ± 11.3 years. The control subjects were chosen to be significantly older than OAG patients with elevated IOP ($P < 0.001$), patients with JOAG ($P < 0.001$), and patients with OAG with normal IOP ($P < 0.001$) to try to reduce the likelihood that control subjects would eventually have glaucoma.

The clinical characteristics recorded in patients with glaucoma were age at diagnosis, untreated maximum IOP (defined as IOP at diagnosis), and visual field defects at the initial examination (defined as visual field defects at diagnosis). The mean age at diagnosis was 58.0 ± 12.1 years in patients with OAG with elevated IOP, 26.4 ± 6.0 years in patients with JOAG, and 57.4 ± 11.5 years in patients with OAG with normal IOP. The mean IOP at diagnosis was 26.7 ± 6.4 mm Hg in the patients with OAG with elevated IOP, 31.0 ± 10.7 mm Hg in patients with JOAG, and 16.6 ± 2.4 mm Hg in patients with OAG with normal IOP. The mean visual field score at diagnosis was 5.1 ± 0.9 in patients with OAG with elevated IOP, 3.0 ± 0.8 in patients with JOAG, and 2.8 ± 0.7 in patients with OAG with normal IOP. A positive family history was recorded in 68 (31.6%) of 215 patients with OAG with elevated IOP, in 13 (34.2%) of 38 patients with JOAG, and in 86 (31.0%) of 277 patients with OAG with normal IOP. There were 124 (57.7%) men in the patients with OAG with elevated IOP, 27 (71.1%) in the patients with JOAG, 132 (47.7%) in the patients with OAG with normal IOP, and 114 (47.5%) in the control group.

The severity of the visual field defects was scored from 1 to 5 according to previously reported criteria.^{32,33} The data obtained by two types of perimeters were combined by using a five-point scale: 1, no alterations; 2, early defects; 3, moderate defects; 4, severe defects; and 5, light perception only or no light perception. The first four groups on this severity scale followed Kozaki's classification based on Goldmann perimetry,^{33,34} or the classification was based on the results of visual field perimetry (Humphrey Field Analyzer; Carl Zeiss Meditec, Dublin, CA).³⁵ Kozaki's classification is widely used in Japan.

DHPLC Analysis

Genomic DNA was isolated from peripheral blood lymphocytes by phenol-chloroform extraction. The six exonic coding regions of the *noelin 2* gene were amplified by polymerase chain reaction (PCR) using the primer sets listed in Table 1. Only exon 1 was screened by direct sequencing. The other exons were screened by DHPLC analysis (Wave System; Transgenomic, Omaha, NE). A detailed description of the DHPLC technique has been published.³⁰ The temperatures for analysis of the DNA fragments are listed in Table 1. During the high-throughput analysis by DHPLC, samples from three patients were pooled, and PCR was performed as previously described.³⁰ When the chromatographic patterns differed from control patterns in the pooled samples according to the high-throughput protocol, the samples were reanalyzed individually. PCR products that showed different chromatographic patterns from control were then sequenced. The exon 4 region of *OLFM2* was examined in 959 blood samples (770 blood samples and an additional 189 samples); 280 patients with OAG with elevated IOP, 341 patients with OAG with normal IOP, 38 patients with JOAG, and 300 control subjects.

Direct Sequencing of DNA

After purification of the PCR products, sequencing reactions were performed using dyc termination chemistry (Prism BigDye Terminator, ver. 3.1 Cycle Sequencing Kit; Applied Biosystems [ABI], Foster City, CA), according to the manufacturer's protocol. The data were collected by a gene analyzer (Prism 310; ABI) and analyzed by computer (PRISM Sequencing Analysis Program, ver. 3.7; ABI).

Genotyping SNPs

Genotyping of c.315G→A (Ala105Ala), c.317G→A (Arg106Gln), c.451G→A (Arg144Gln), c.678G→A (Ala226Ala), and c.1281C→T (Arg427Arg) in the *noelin 2* gene, and genotyping of c.412G→A (Thr54Thr) and c.603T→A (Met98Lys) in the optineurin gene were confirmed by the chromatographic pattern of DHPLC and restriction

TABLE 1. Primer Sequences, PCR Product Sizes, and PCR Annealing and DHPLC Analysis Temperatures for Noelin 2 Gene

Exon	Area	Primer Sequences (5' to 3')	PCR Product Size (bp)	PCR Tm (°C)	DHPLC Tm (°C)
1		F GCAACAAAGACTCGGAGCGA R CTCGCCAACCCGCTTTCTG	335	55	not performed
2		F GCGAGACCCCTCATCGGAGATT R CCGTGGAGAGGACCTGGATT	344	67	62.0, 63.0, 64.0
3		F CGTTGGGATTTGGGGAAGGA R CCAGACATGACTCGATTGTAGGAA	284	67	60.3, 62.3, 64.3
4	A	F GAGTCAGAGGTTGGAGTCATGT R CGTTGGTGCAGGTCCTCATATA	249	65	62.7, 63.2, 63.7
	B	F CAGACACGGGGACCATTTGTA R GGGTGTGGCAGTCAGAGATCA	208	65	63.1, 64.1, 65.1
5		F CCGAACCTTGATCAGCAGGACTT R CTAGGCACTTATGGGAGTCAA	269	65	61.7, 63.7, 64.7
6	A	F CTAATGGCTGTAGCTGGTGCT R CTAGGGGGAAGGTGTTGTTAA	336	65	62.5, 63.5, 64.5
	B	F CCAGAGCAACCGTGGTGCTGA R CTGGTAACTGGAGGTTGTTGGT	364	65	63.7, 64.7, 65.7
	C	F CATGATCTGGGTGTGCTCTA R GCAGCCGAGCCAGCAGGATT	267	67	61.5, 62.0

enzyme assay (Table 2). The G-to-A substitution at position 227 (Met76Lys) in exon 1 of the myocilin gene was confirmed by the chromatographic pattern of DHPLC and assay³⁶ (Invader assay; provided by the Research Department of R&D Center; BML, Saitama, Japan).

Analysis of Gene-Gene Interaction Using Common SNPs

For the analysis of gene-gene interaction of *MYOC* and *OLFEM2*, or *OPTN* and *OLFEM2* in patients with OAG, the genotypes were defined as follows: *MYOC*, c.227G→A (wild:G, mutant:A); *OPTN*, c.412G→A (wild:G, mutant:A) and c.603T→A (wild:T, mutant:A); and *OLFEM2*, c.317G→A (wild:G, mutant:A), c.678G→A (wild:G, mutant:A), and c.1281C→T (wild:C, mutant:T) (Table 3). The interactions between SNPs were classified in four groups using a dominant model. The genotypes were MM, mutant homozygote; MW, heterozygote; and WW, wild homozygote. In the explanatory variable, group 0 was the reference.

Statistical Analysis

Statistical analysis was performed (SPSS; SPSS Inc., Chicago, IL) and Hardy-Weinberg equilibrium for each SNP was determined by χ^2 test with 1 degree of freedom. After equilibrium was confirmed, the frequencies of the genotypes and alleles of patients with glaucoma were compared with those of control subjects by χ^2 test. Logistic regression analysis was used to calculate age-adjusted odds ratios (ORs) and 95% confidence intervals (CIs), to search for gene-gene interactions in patients with OAG with elevated or normal IOP. As the sample number of the JOAG group was insufficient to attain a normal distribution,

patients with JOAG were excluded from the analysis. The Bonferroni method was used to adjust for multiple comparisons.

Comparisons of the clinical characteristics (age, IOP, and visual field defect at the time of diagnosis) in patients with OAG between the two genotypes in a single gene or between the combined genotypes in the two genes were performed using the Mann-Whitney test or one-way ANOVA, respectively. To adjust for multiple comparisons when one-way ANOVA showed a significant difference between groups ($P < 0.05$), the Tukey honestly significant difference (HSD) adjustment for multiple comparisons was used. $P < 0.05$ was considered significant.

Bioinformatics Analysis

To assess whether the normal residues at disease-causing mutation sites in the noelin 2 gene are conserved across species, the following mRNA entries were selected from National Center for Biotechnology Information (NCBI; Bethesda, MD) web sites: *Danio rerio* (BC044164), *Mus musculus* (NM_173777), *Rattus norvegicus* (NM_001015017), *Macaca fascicularis* (AY650384), and *Homo sapiens* (NM_058164). The sequences were aligned by the multiple alignment tool ClustalW³⁷ (available online at <http://www.dclib.nig.ac.jp/> European Bioinformatics Institute, European Molecular Biology Laboratory, Heidelberg, Germany).

RESULTS

Univariate Analysis of Individual Polymorphisms in *OLFEM2*

The results of the sequence variants are summarized in Table 4. A total of 770 Japanese subjects were studied for all exons, and

TABLE 2. Genotyping *OLFEM2* and *OPTN* Sequence Variants

Gene	Location	Sequence Changes	PCR Product Size (bp)	Genotype (size in bp)		Restriction Enzyme
				Wild Homozygote	Mutant Homozygote	
<i>OLFEM2</i>	Exon 3	c.315G→A	284	c.315G (284)	c.315A (152, 132)	<i>Hpy</i> CH ₁ V
	Exon 3	c.317G→A	284	c.317G (284)	c.317A (153, 131)	<i>Fsp</i> I
	Exon 4	c.431G→A	344	c.431G (280, 144)	c.431A (344)	<i>Bst</i> UI
	Exon 5	c.678G→A	269	c.678G (269)	c.678A (185, 84)	<i>Hpy</i> CH ₁ V
	Exon 6	c.1281C→T	267	c.1281C (157, 110)	c.1281T (267)	<i>Bss</i> IIII
	Exon 4	c.412G→A	317	c.412G (188, 129)	c.412A (317)	<i>Hpy</i> CH ₁ IV
<i>OPTN</i>	Exon 5	c.603T→A	277	c.603T (277)	c.603A (175, 102)	<i>Stu</i> I

TABLE 3. Combination of Genotype between Two Genes

	Genotype*	SNPs in <i>OLFM2</i> †	
		MM and MW	WW
SNPs in <i>OPTN</i> ‡ or <i>MYOC</i> §	MM and MW	3	1
	WW	2	0

* MM is mutant homozygote, MW is heterozygote, and WW is wild homozygote.

[†] c.317G→A (W/G, M/A), c.678G→A (W/G, M/A) and c.1281C→T (W/C, M/T) in *OLFMT2*.

[‡] c.412G→A (W/G, M/A), and c.603T→A (W/T, M/A) in *OPTN*.

[§] c.227G→A (W/G, M/A) in *MYOC*.

an additional 189 subjects also were studied for Arg144Gln in exon 4 for a total of 959 subjects in all. Twelve sequence variants of the noelin 2 gene were identified. Two variants, c.317G→A (Arg106Gln) and c.1281C→T (Arg427Arg), were reported as haplotype markers in the international HapMap Project, and 10 variants were novel: c.431G→A (Arg144Gln); c.622C→T (Arg208Trp); c.258G→A (Thr86Thr); c.315G→A (Ala105Ala); c.420G→A (Lys140Lys); c.456G→A (Glu152Glu); c.597→T (Thr199Thr); c.678G→A (Ala226Ala); c.360+34_37delCAAA; and c.1365+39T→C. Among the sequence changes, three were missense changes, seven were synonymous codon changes, and two were changes in the noncoding sequences.

The missense change, Arg144Gln, located within the exon 4 myosin-like domain, was found in only two patients with OAG: one with elevated and one with normal IOP. The patient with OAG with elevated IOP (maximum, 29 mm Hg in the right eye and 32 mm Hg in the left) had central corneal thickness (CCT) of 499 μ m in the right eye and 498 μ m in the left. The patient with OAG with normal IOP (maximum, 18 mm Hg in both eyes) had CCT of 521 μ m in the right eye and 549 μ m in the left. The Arg144Gln amino acid change was a possible disease-causing mutation. Four patients with *MYOC* mutations and two patients with *OPTN* mutation did not harbor the Arg144Gln mutation in *OLFMT2*.

Three common SNPs, c.317G→A (Arg106Gln), c.678G→A (Ala226Ala), and c.1281C→T (Arg427Arg) followed the Hardy-Weinberg equilibrium. There was no significant association of the three SNPs with glaucoma ($P > 0.017$, the Bonferroni-corrected significance level). The genotypes of the three common SNPs were not significantly associated with the clinical characteristics based on this analysis.

Bioinformatic Analysis of the Disease-Causing Mutation Arg144Gln

The normal residue at the mutation site of Arg144 in the noelin 2 gene was highly conserved across species including *Homo sapiens*, *Macaca fascicularis*, *Rattus norvegicus*, *Mus musculus*, and *Danio rerio* (Fig. 1).

Distribution of Thr341Thr and Met98Lys in *OPTN* and Arg76Lys in *MYOC* in Patients and Control Subjects

A total of 770 Japanese subjects were studied, which was higher than the number in a previous study of *OPTN*.³⁰ The observed genotype frequency was in agreement with those predicted by the Hardy-Weinberg equilibrium. The c.412G→A (Thr341Thr) was weakly associated with patients with OAG with elevated IOP ($P = 0.030$ for allele frequency; Table 4). In contrast, the c.603T→A (Met98Lys) was weakly associated

TABLE 4. *OLFMT2*, *OPTN*, and *MYOC* Variants Observed in Patients with Glaucoma Control Subjects

Gene	Location	Sequence Changes	Amino Acid Changes	Allele Frequency in Subjects (%)				Genotype Frequency in Subjects (%)			
				JOAG (n = 76)	OAG with Elevated IOP (n = 436)	OAG with Normal IOP (n = 554)	Control (n = 480)	JOAG (n = 38)	OAG with Elevated IOP (n = 215)	OAG with Normal IOP (n = 277)	Control (n = 240)
<i>OLFMT2</i>	Exon 3	c.317G→A	Arg106Gln [‡]	24 (31.6)	132 (30.7)	168 (30.3)	134 (27.9)	4/16 (19%)	109 (51.0)	32/104 (41)	19/96 (12)
	Exon 3	c.622C→T	Arg208Trp	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Exon 3	c.258G→A	Thr86Thr	0 (0.0)	1 (0.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Exon 3	c.315G→A	Ala105Ala	0 (0.0)	1 (0.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Exon 4	c.420G→A	Lys140Lys	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Exon 4	c.456G→A	Glu152Glu	0 (0.0)	2 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Exon 5	c.597G→T	Thr199Thr	0 (0.0)	0 (0.0)	1 (0.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Exon 5	c.678G→A	Ala226Ala	3 (3.9)	16 (3.7)	28 (5.1)	28 (5.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Exon 6	c.1281C→T	Arg427Arg	3 (3.9)	35 (8.1)	46 (8.3)	39 (8.1)	0 (0.0)	1/33 (18)	1/42 (2)	0 (0.0)
	Intergenic	c.360+34_37delCAAA	—	42 (60.0)	281 (66.0)	93 (16.8)	66 (13.8)	10/22 (3)	9/69 (13)	9/69 (13)	NC [§]
<i>OPTN</i>	Exon 4	c.412G→A	Thr341Thr	16 (21.6)	62 (14.6)	93 (16.8)	66 (13.8)	0 (0.0)	8/77 (10)	3/60 (5)	3/60 (5)
	Exon 5	c.603T→A	Met98Lys	6 (8.3)	38 (8.8)	66 (11.9) [†]	38 (7.9)	0 (0.0)	1/36 (2)	1/36 (2)	0 (0.0)
<i>MYOC</i>	Exon 1	c.227G→A	Arg76Lys	4 (5.3)	17 (3.9)	28 (5.1)	17 (3.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

* n = 560 in OAG with elevated IOP, n = 682 in OAG with normal IOP, and n = 900 in Control for Arg144Gln, Lys140Lys, Glu152Gln.

[†] n = 280 in OAG with elevated IOP, n = 351 in OAG with normal IOP, and n = 300 in Control for Arg144Gln, Lys140Lys, Glu152Gln.

[‡] These codon changes had already reported as haplotype markers in the international HapMap Project (c.317G→A, Arg106Gln; c.622C→T, Arg208Trp; c.258G→A, Thr86Thr; c.315G→A, Ala105Ala; c.420G→A, Lys140Lys; c.456G→A, Glu152Glu; c.597G→T, Thr199Thr; c.678G→A, Ala226Ala; c.360+34_37delCAAA; and c.1365+39T→C).

[§] NC, not checked.

^{||} P < 0.05.

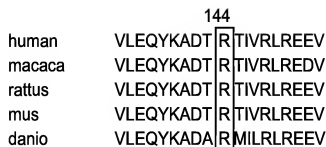


FIGURE 1. Sequence alignment of mutant amino acid in *OLFM2* for *Homo sapiens* (human), *Macaca fascicularis* (macaca), *Rattus norvegicus* (rattus), *Mus musculus* (mus), and *Danio rerio* (danio). Alignment of amino acids was performed using the ClustalW alignment tool. Arg(R)144 was conserved among these species.

with patients with OAG with normal IOP ($P = 0.033$ for allele frequency).

No significant difference was detected between patients with glaucoma and control subjects with respect to genotypes or allele frequency of c.227G→A (Arg76Lys) in *MYOC*.

Analysis of Gene-Gene Interactions in Patients with OAG

For analysis of gene-gene interactions using the three common SNPs in *OLFM2* and one common SNP of c.412G→A (Thr34Thr) in *OPTN*, a logistic regression model was built (Table 5). A significant association was found in the explanatory variable type 3 in this gene-gene interaction. *OLFM2*/317A and *OPTN*/412A and the *OLFM2*/1281T and *OPTN*/412A mutations were significantly associated with OAG with elevated IOP ($P = 0.018$, OR = 2.232, 95% CI: 1.150–4.331; $P = 0.012$, OR = 4.240, 95% CI: 1.369–13.137, respectively).

For analysis of gene-gene interactions using the three common SNPs in *OLFM2* and one common SNP of c.603T→A (Met98Lys) in *OPTN*, a logistic regression model was built (Table 6). A significant association was found in the explanatory

tory variable type 1 in this gene-gene interaction. *OLFM2*/non-317A and *OPTN*/603A were significantly associated with OAG with elevated IOP ($P = 0.018$, OR = 2.572, 95% CI: 1.173–5.642), and *OLFM2*/non-678A and *OPTN*/603A were weakly associated with OAG with normal IOP ($P = 0.038$, OR = 1.808, 95% CI: 1.034–3.162).

No significant interaction was detected between the three SNPs in *OLFM2* and one common SNP of c.227G→A (Arg76Lys) in *MYOC* in patients with glaucoma (data not shown).

Comparison of Clinical Characteristics of Patients with Glaucoma with the Combined Genotypes in the Two Genes

Comparison analyses of three phenotypic variables in patients with OAG with elevated IOP or with normal IOP in association with three common SNPs of c.317G→A (Arg106Gln), c.678G→A (Ala226Ala), and c.1281C→T (Arg427Arg) in *OLFM2* did not show any statistical significance (data not shown).

To examine the effect of combined genotypes in the two genes on considered phenotypic variables, three SNPs (Arg106Gln, Ala226Ala, and Arg427Arg) in *OLFM2* were combined with two SNPs (Thr34Thr and Met98Lys) in *OPTN* or one SNP (Arg76Lys) in *MYOC*. Thus, nine combined genotypes in the two genes were obtained. The associations between the nine combined genotypes in the two genes and the phenotypic variables (age, IOP, and visual field score) at the time of diagnosis of OAG in patients with elevated IOP or with normal IOP were determined by one-way ANOVA. Among the nine, the two combined genotypes of Ala226Ala+Thr34Thr (or c.678G→A+c.412G→A) and Arg427Arg+Thr34Thr (or c.1281C→T+c.412G→A) showed statistical significance in patients with OAG with normal IOP ($P = 0.036$ and $P = 0.039$, respectively) in the visual field score. Patients with OAG with normal IOP who were *OLFM2*/678A and *OPTN*/412G carriers had worse visual field scores ($P = 0.022$) than those who were *OLFM2*/678G and *OPTN*/412A carriers based on the Tukey

TABLE 5. Analysis of Gene-Gene Interactions Using Common Polymorphisms of *OLFM2* and *OPTN*(c.412G→A)

SNP in Gene		Phenotype	Explanatory Variable	Regression Coefficient B	Odds Ratio Exp (B)	95% CI	P*
<i>OLFM2</i>	<i>OPTN</i>						
c.317G→A (Arg106Gln)	c.412G→A (Thr34Thr)	OAG with elevated IOP	3	0.803	2.232	1.150–4.331	0.018†
			2	−0.139			
			1	0.056			
		OAG with normal IOP	3	0.415			
			2	−0.192			
			1	−0.231			
c.678G→A (Ala226Ala)	c.412G→A (Thr34Thr)	OAG with elevated IOP	3	0.252	4.240	1.369–13.137	0.012†
			2	−0.337			
			1	0.435			
		OAG with normal IOP	3	0.644			
			2	−0.496			
			1	0.042			
c.1281C→T (Arg427Arg)	c.412G→A (Thr34Thr)	OAG with elevated IOP	3	1.445	0.160		0.498
			2	0.269			
			1	0.375			
		OAG with normal IOP	3	0.621			
			2	0.368			
			1	0.160			

* P by logistic regression analysis.

† P < 0.05.

TABLE 6. Analysis of Gene-Gene Interactions Using Common Polymorphisms of *OLFM2* and *OPTN*(c.603T>A)

SNP in Gene		Phenotype	Explanatory Variable	Regression Coefficient B	Odds Ratio Exp (B)	95% CI	P*
<i>OLFM2</i>	<i>OPTN</i>						
c.317G→A (Arg106Gln)	c.603T→A (Met98Lys)	OAG with elevated IOP	3	0.035	2.572	1.173–5.642	0.932
			2	0.356			0.127
			1	0.945			0.018†
		OAG with normal IOP	3	0.311			0.369
			2	0.100			0.654
			1	0.484			0.218
c.678G→A (Ala226Ala)	c.603T→A (Met98Lys)	OAG with elevated IOP	3	-1.795	1.808	1.034–3.162	0.107
			2	-0.012			0.976
			1	0.588			0.054
		OAG with normal IOP	3	-1.008			0.149
			2	0.089			0.809
			1	0.592			0.038†
c.1281C→T (Arg427Arg)	c.603T→A (Met98Lys)	OAG with elevated IOP	3	0.914	1.808	1.034–3.162	0.122
			2	0.388			0.271
			1	0.281			0.377
		OAG with normal IOP	3	0.161			0.792
			2	0.547			0.094
			1	0.511			0.075

* P by logistic regression analysis.

† P < 0.05.

HSD (Table 7). Patients with OAG with normal IOP who were *OLFM2*/1281C and *OPTN*/412G carriers had worse visual field scores ($P = 0.030$) than those who were *OLFM2*/1281T and *OPTN*/412A carriers based on the Tukey HSD.

DISCUSSION

The phylogenetic analysis suggested that myocilin may have evolved from *OLFM2* by gene duplication followed by exon fusion: The gene was composed of a myosin-like domain (exons 1, 2, 3, and 4) and an olfactomedin-like domain (exons 5 and 6).^{24,25} We screened *OLFM2* in Japanese patients with OAG and normal subjects to identify disease-causing mutations. Twelve sequence variants were identified: Two (Arg106Gln and Arg427Arg) have been reported and 10 were novel. The Arg144Gln found in exon 4 of the myosin-like domain was detected exclusively in two patients with OAG with elevated IOP and with normal IOP (0.3%, 2/659), and was not detected in 300 normal subjects. Fingert et al.¹⁴ set the criteria for probable disease-causing mutations in the myocilin gene: (1) altered myocilin amino acid sequence; (2) the presence of the mutation in one or more

patients with glaucoma; (3) presence of the mutation in less than 1% of the general population; and (4) absence of the mutation in normal individuals. The Arg144Gln mutation matched all four criteria.

We also assessed the normal residues at disease-causing mutation sites in noelin 2 by ClustalW. Arg144 was highly conserved across species, including *Homo sapiens*, *Macaca fascicularis*, *Rattus norvegicus*, *Mus musculus*, and *Danio rerio* (Fig. 1). From the criteria for mutations and highly conserved sequences at the mutation site, we concluded that the Arg144Gln change is a disease-causing mutation. Arginine is a basic amino acid and is positively charged. In contrast, glutamine is an acidic, polar uncharged amino acid. Four patients with *MYOC* mutations and two patients with *OPTN* mutation did not harbor the Arg144Gln mutation in *OLFM2*. There were no significant differences in allele frequency and genotype frequency between the patients with glaucoma and the normal subjects in the three common SNP groups of the noelin 2 gene: Arg106Gln, Ala226Ala, and Arg427Arg (Table 4).

Interaction analyses of the noelin 2, myocilin, and optineurin genes were performed in patients with OAG by using a logistic regression model based on the combinations of com-

TABLE 7. Comparison of Clinical Characteristics of Patients with Glaucoma Associated with Interactions of Two Genes

Phenotype	Phenotype Variable	<i>OLFM2</i> /678G + <i>OPTN</i> /412A	<i>OLFM2</i> /678A + <i>OPTN</i> /412G	P*
OAG with normal IOP	Age at diagnosis (y)	57.2 ± 11.3 (n = 74)	55.2 ± 13.5 (n = 17)	NC†
	IOP at diagnosis (mm Hg)	16.5 ± 2.2 (n = 70)	15.9 ± 3.0 (n = 14)	NC†
	Visual field score	2.7 ± 0.6 (n = 74)	3.2 ± 0.8 (n = 17)	0.022†
Phenotype	Phenotype Variable	<i>OLFM2</i> /1281T + <i>OPTN</i> /412A	<i>OLFM2</i> /1281C + <i>OPTN</i> /412G	P*
OAG with normal IOP	Age at diagnosis (y)	57.3 ± 7.6 (n = 11)	57.9 ± 11.5 (n = 158)	NC†
	IOP at diagnosis (mm Hg)	16.4 ± 2.2 (n = 11)	16.6 ± 2.6 (n = 141)	NC†
	Visual field score	2.3 ± 0.5 (n = 11)	2.9 ± 0.7 (n = 158)	0.030†

* P by multiple comparison (Tukey HSD).

† P < 0.05.

‡ NC, not calculated. One-way ANOVA did not show a significant difference between groups ($P > 0.05$).

mon SNPs in the two genes. A significant association was found in the explanatory variable type 3 in two SNPs (Arg106Gln and Arg427Arg) in *OLF2* and Thr34Thr in *OPTN*, and in variable type 1 in two SNPs (Arg106Gln and Ala226Ala) in *OLF2* and Met98Lys in *OPTN*. These results suggest that common SNPs in *OLF2* and *OPTN* contribute interactively to OAG, indicating a polygenic etiology with different properties for Thr34Thr and Met98Lys in *OPTN*.

In vivo experiments using rats, protein-protein interaction between optineurin and myocilin through the conserved olfactomedin domain has been demonstrated.²³ In the present study, however, no significant difference was detected in gene-gene interaction using three SNPs in *OLF2* and one SNP for c.227G→A (Arg76Lys) in *MYOC*. Arg76Lys is the only common polymorphism reported in the Japanese population. If other common SNPs in *MYOC* are identified in the Japanese population, gene-gene interactions may be identified.

Comparison analysis of three phenotypic variables of patients with OAG with elevated IOP or with normal IOP in association with three SNPs of c.317G→A (Arg106Gln), c.678G→A (Ala226Ala), and c.1281C→T (Arg427Arg) in *OLF2* did not show any statistical significance. However, the patients with OAG with normal IOP who were *OLF2*/678A and *OPTN*/412G carriers had significantly worse visual field scores ($P = 0.022$) than those who were *OLF2*/678G and *OPTN*/412A carriers. Furthermore, the patients with OAG with normal IOP who were *OLF2*/1281C and *OPTN*/412G carriers had significantly worse visual field scores ($P = 0.030$) than those who were *OLF2*/1281T and *OPTN*/412A carriers. These results suggest that the two genes may contribute interactively to clinical features in patients with OAG, indicating a polygenic etiology. We have reported possible gene-gene interactions between common SNPs in *OPTN* and *TNF4* for Japanese patients with OAG by genetic statistical analysis.³⁰ In Chinese patients with POAG, possible interactions were also reported between common SNPs in *MYOC*, *OPTN*, and *APOE*.²⁸ In view of the polygenic nature of OAG, it is quite important to explore possible gene-gene interactions between known candidate genes. This type of study will provide valuable clues for further functional studies to decipher the complex pathogenesis of glaucoma.

Recent investigations have disclosed that people classified as having ocular hypertension have thicker central corneal thickness (CCT) than do control subjects,³⁸⁻⁴² whereas those with normal-tension glaucoma have thinner CCT.³⁹ Unfortunately, the CCT was not measured in all subjects enrolled. The CCT must be considered when developing a treatment approach for patients with ocular hypertension.³⁸⁻⁴⁰ However, the epidemiologic study of glaucoma in a Japanese population, the Tajimi study, showed no significant intergroup difference in CCT among OAG with IOP > 21 mm Hg (523 ± 35 μ m), OAG with IOP ≤ 21 mm Hg (518 ± 29 μ m), and subjects without glaucoma (520 ± 32 μ m), when measured by a noncontact specular-type instrument (SP-2000P; Topcon, Tokyo, Japan).⁴¹ In the normal Japanese population in the Tajimi study, the IOP measured with Goldmann applanation tonometry correlated positively with CCT, with corrected IOP (IOP reading $-0.012 \times [\text{CCT (in } \mu\text{m)} - 520]$).⁴² This formula suggests that a difference in CCT of 50 μ m necessitates adjustment of IOP of 0.6 mm Hg in the Japanese population.

In our study, the mean IOP at diagnosis was 26.7 ± 6.4 mm Hg in the 215 patients with OAG with elevated IOP and 16.6 ± 2.4 mm Hg in the 277 patients with OAG with normal IOP. For each patient with OAG, the CCT must be considered to make an exact clinical definition of the two types. Some of the patients with OAG with borderline IOP around 21 ($n = 4$) or 22 mm Hg ($n = 15$) in the present study may change their clinical definition of the two types, depending on CCT. How-

ever, considering that the number of patients with OAG with borderline IOP around 21 or 22 mm Hg was small and that adjustment for IOP with CCT (0.6 mm Hg/50 μ m) would be small in our Japanese population, the CCT would most likely not significantly influence our statistical analysis of association studies in Tables 5 and 6 in 770 subjects.

In conclusion, the Arg144Gln mutation in the *noelin 2* gene is a possible disease-causing mutation in Japanese patients with OAG. Common SNPs in *OLF2* and *OPTN* may interactively contribute to OAG, indicating a polygenic etiology.

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